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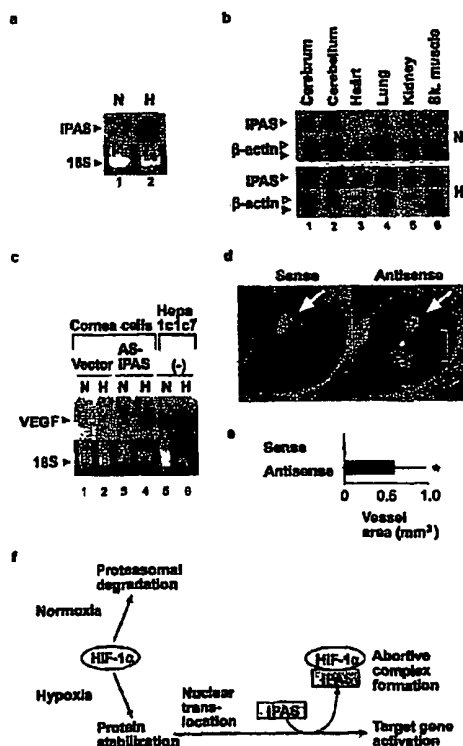
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(54) Title: REGULATION OF HYPOXIA-INDUCIBLE GENE EXPRESSION WITH ANTISENSE INHIBITORY PAS DOMAIN PROTEIN



(57) Abstract: A pharmaceutical composition comprises the sequence of SEQ ID NO:s 3-5 or pharmaceutically equivalent sequences. Methods employing the pharmaceutical compositions include treatments to increase angiogenesis, to stimulate HIF-1α function, to treat hypoxia-related conditions, and to maintain normal cell functions under hypoxia.



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REGULATION OF HYPOXIA-INDUCIBLE GENE EXPRESSION WITH ANTISENSE INHIBITORY PAS DOMAIN PROTEIN

FIELD OF THE INVENTION

The invention relates generally to the field of proteins related to angiogenic processes. The invention further relates to methods useful for inducing angiogenesis.

BACKGROUND OF THE INVENTION

Angiogenesis is a critical function for normal organism functioning and survival. Insufficient angiogenesis can result in cell or tissue death. Excessive angiogenesis has been linked to tumor development and other undesirable conditions. Some of the genes and biochemical processes involved in angiogenesis have been described. For example, dysregulation or enhanced activation of HIF-1 α function have been implicated in a variety of pathological conditions including induced tumor growth rates^{9,17,18} and inflammatory angiogenesis¹⁹. Since it is important for organisms to maintain normal angiogenesis and for certain cells or tissues to be able to functionally counteract the activated form of HIF-1 α , there remains a need in the art to provide both further information on the mechanisms that underlie angiogenesis and to present new methods useful in either increasing or decreasing angiogenesis.

SUMMARY OF THE INVENTION

It is therefore an object of the present invention to present further information related to angiogenesis and to supply novel methods to affect angiogenic responses.

According to a first embodiment of the invention, a pharmaceutical composition is provided which comprises the sequence of SEQ ID NOS:3, 4, 5, sequences complementary to SEQ ID NO:1 or active fragments or analogs thereof. The composition may further comprise pharmaceutical carriers, adjuvants, and diluents.

According to a further embodiment of the invention, methods of increasing angiogenesis, methods of increasing HIF-1 α function, methods of treating a hypoxia-related condition, and methods of maintaining normal cell or tissue function under hypoxic conditions in a cell, a group of cells, or an organism are provided, which

comprise administering a pharmaceutically effective amount of a composition according to the first embodiment to the cell, group of cells, or organism. The hypoxia-related condition may be, for example, ischemia, coronary heart disease, wound healing, stroke, or diabetic ulceration.

According to a further embodiment of the invention, a plasmid is provided comprising the sequence of SEQ ID NOS:3, 4, 5, or sequences complimentary to SEQ ID NO:1. The invention also includes vectors containing such a plasmid and host cells transfected with such a vector.

According to a further embodiment of the invention, a host cell is provided which expresses the protein encoded by any one of SEQ ID NOS:3, 4, or 5.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows the deduced amino acid sequence of mouse IPAS (SEQ ID NO:1);

Figure 1B is a diagram representing a comparison between mouse IPAS, mouse HIF-1 α , and mouse HLF/EPAS1 with percent identities;

Figure 1C shows the results of a RNA blot analysis of IPAS expression in adult mice tissues;

Figure 2 shows the results of an *in situ* hybridization analysis of IPAS expression in selected regions of adult mice, specifically,

Figure 2A shows a light field section of cornea after hybridization with antisense RNA probes of mIPAS;

Figure 2B shows a dark field section of cornea after hybridization with antisense RNA probes of mIPAS;

Figure 2C shows a light field section of cornea after hybridization with antisense RNA probes of mHIF-1 α ;

Figure 2D shows a dark field section of cornea after hybridization with antisense RNA probes of mHIF-1 α ;

Figure 2E shows a light field section of retina after hybridization with antisense RNA probes of mIPAS;

Figure 2F shows a dark field section of retina after hybridization with antisense RNA probes of mIPAS;

Figure 2G shows a light field section of retina after hybridization with antisense RNA

probes of mHIF-1 α ;

Figure 2H shows a dark field section of retina after hybridization with antisense RNA probes of mHIF-1 α ;

Figure 2I shows a light field section of cerebellum after hybridization with antisense RNA probes of mIPAS;

Figure 2J shows a dark field section of cerebellum after hybridization with antisense RNA probes of mIPAS;

Figure 2K shows a light field section of cerebellum after hybridization with antisense RNA probes of mHIF-1 α ;

Figure 2L shows a dark field section of cerebellum after hybridization with antisense RNA probes of mHIF-1 α ;

Figure 3A shows IPAS inhibition of HIF mediated reporter gene activation in hypoxic HeLa cells;

Figure 3B shows IPAS inhibition of HLF/EPAS1 mediated reporter gene activation in hypoxic HeLa cells;

Figure 3C shows that HIF-1 α and HLF protein stability are not affected by IPAS in HeLa cells;

Figure 3D demonstrates hypoxia-inducible gene expression impairment by IPAS overexpressing cells;

Figure 3E shows IPAS inhibition of the DNA binding activity of HIF-1 α , where the asterisk indicates constitutive HRE binding activity, the arrow indicates a HIF-1 α /Arnt-DNA complex, S indicates unlabeled HRE control and NS indicates unrelated sequence control;

Figure 3F shows immunoprecipitation with anti-GST antibodies revealing that GST IPAS physically interacts with *in vitro* translated HIF-1 α ;

Figure 3G represents a coimmunoprecipitation of ³⁵S-labeled IPAS with GAL4-fused HIF-1 α fragments by anti-GAL4 antibodies showing the N-terminus of HIF-1 α mediating heterodimerisation with IPAS;

Figure 3H shows how combinations of *in vitro* translated IPAS, HIF-1 α , Arnt, or control lysate were analyzed to reveal that the IPAS/HIF-1 α heterodimer fails to bind the HRE sequence motif;

Figure 4 shows IPAS expression in hepatoma cells retards tumor growth, specifically,

Figure 4A shows increases in tumor volumes after subcutaneous implantation of wild-type Hepalclc7 or Hepa IPAS cells in immunodeficient SCID mice, where the asterisk indicates ($p < 0.001$) difference in results between the two cell lines and a double asterisk indicates a difference of ($p < 0.01$);

Figure 4B shows vascular density by immunohistochemical staining for CD31 antigen expression

Figure 4C represents a numerical analysis of vascular density with results obtained by counting under a light microscope, where an asterisk indicates ($p < 0.001$) difference between wild-type and cell derived tumors;

Figure 5A shows IPAS expression in primary cultures of mouse cornea epithelium cells using RNA blotting following incubation under normoxic or hypoxic conditions for 24 hours;

Figure 5B shows hypoxia inducible expression of IPAS mRNA in mouse tissues, where the white arrow indicates a 2.0 kb form of β -actin and a black arrow indicates a 1.8 kb form of β -actin;

Figure 5C shows VEGF mRNA expression in hepatoma or primary cornea cells transfected with control or antisense IPAS vectors;

Figure 5D displays photographs of the results of ocular angiogenesis studies wherein polymers containing IPAS antisense or sense oligonucleotides were implanted in to micropockets of the cornea, the area represented by an arrow, showing the antisense oligonucleotides caused neovascularization as indicated by the bracket;

Figure 5E results of ocular angiogenesis studies presenting the mean \pm SD area of neovascularisation, where an asterisk represents a difference of ($p < 0.001$) between the scrambled and sense oligonucleotide treated groups;

Figure 5F is a schematic model of the negative regulation of HIF-1 α function by IPAS; and

Figure 6 shows the full length IPAS DNA sequence (SEQ ID NO:2).

DETAILED DESCRIPTION

Alteration of gene expression is a crucial component of adaptive responses to hypoxia. These responses are mediated by hypoxia-inducible transcription factors (HIFs)^{1,2}. A novel basic helix-loop-helix (bHLH)/PAS protein related to HIFs was

identified by methods detailed below and termed Inhibitory PAS (Per/Arnt/Sim) domain protein, or IPAS. IPAS was found to contain no transactivation function and demonstrated dominant negative regulation of HIF-mediated control of gene expression. Ectopic expression of IPAS in hepatoma cells selectively impaired induction of genes involved in adaptation to a hypoxic environment, notably the vascular endothelial growth factor (VEGF) gene. It also resulted in retarded tumor growth and reduced tumor vascular density *in vivo*.

IPAS shows predominantly nuclear localization under both normoxic and hypoxic conditions, as assessed by green fluorescent fusion protein assays (data not shown). As described more fully below, IPAS was predominantly expressed in mice in Purkinje cells of the cerebellum and in corneal epithelium of the eye. Expression of IPAS in the cornea correlated with low levels of expression of the VEGF gene under hypoxic conditions. Strikingly, application of an IPAS antisense oligonucleotide to the mouse cornea induced angiogenesis under normoxic conditions, and unmasked hypoxia-dependent induction of VEGF gene expression in hypoxic cornea cells. These unexpected results indicate a novel mechanism for negative regulation of angiogenesis and maintenance of an avascular phenotype.

Example 1: Isolation of IPAS cDNA and Creation of Antisense IPAS Expression

Plasmid

Hidden Markov Model profiles³ for HIF homology were built using the HMMER 1.8.3 software²¹ from nucleotide sequences corresponding to the PAS domain of a selected number of bHLH/PAS factors. The model profiles were used to search mouse expressed sequence tag (EST) databases. An EST clone encoding a putative novel protein and containing a bHLH PAS motif was identified (Gene Bank accession # AA028416) and was subsequently obtained, sequenced and designated IPAS.

The nucleotide sequence for IPAS is provided at SEQ ID NO:2 (Fig. 6). DNA sequence analysis revealed that IPAS cDNA contains an open reading frame of 921 nucleotides, encoding a polypeptide of 307 amino acids (Fig. 1A, SEQ ID NO:1). Alignment analysis of the amino acid sequence with known bHLH PAS factors showed high similarity to HIF-1 α ⁴ and HLF/EPAS1^{5,6} in the N-terminal bHLH domain

(75% and 76% identity, respectively; Fig. 1B), and to a lesser extent within the PAS region (34% and 36% in the PAS A, and 40% and 36% in the PAS B domain, respectively; Fig. 1B). Notably, IPAS was found to lack the sequence corresponding to C-terminal region of HIF-1 α and HLF/EPAS1, in which two transactivation domains (N-TAD and C-TAD) have been previously identified (Fig. 1B).

pcDNA3 IPAS was constructed by inserting an *EcoRI*-*Not* I fragment from pT7T3D IPAS (Gene Bank accession # AA028416) into pcDNA3 (Invitrogen). The resulting vector was then used to subclone IPAS into the pCMV FLAG vector²², pGEX-4T-3 (Amersham-Pharmacia Biotech), pCMX GAL4, or pCMX VP-16, to create expression vectors for GST, GAL4, or VP16 fusion proteins, respectively. Full length IPAS cDNA was inserted in an inverted direction into pcDNA3, constructing an antisense IPAS expression plasmid.

Example 2: Cell Culture and Transfection Experiments

IPAS-expressing HEPA cells (Hepa IPAS cells) were established by stable transfection of mouse hepatoma Hepal1c7 cells (ATCC) with pEFIRESpuro IPAS and puromycin (5 μ g/ml) selection. In luciferase reporter gene assays, 0.5 μ g of reporter plasmid and indicated amounts of expression plasmids were used for transfection by lipofection. Conditions for hypoxia or TCDD treatment of the cells have been previously reported¹⁶. Protein expression was monitored in whole cell extracts by immunoblot assays using HIF-1 α (Novus), HLF/EPAS1 (Novus), and FLAG epitope (Sigma) antibodies essentially as previously reported^{8,22}.

IPAS mRNA expression was not detected in the wild-type cells (Fig. 3D). Under hypoxic conditions these cells showed markedly increased expression of phosphoglycerate kinase1 and VEGF mRNA expression, as expected^{11,12}. In response to hypoxia, Hepa IPAS cells showed decreased levels of hypoxia-dependent activation of these genes (45% and 48% reduction of the induction response, respectively; Fig. 3D). This IPAS-mediated effect was at the transcriptional level, since activation of a transiently transfected HRE-driven reporter gene by hypoxia was significantly lower in Hepa IPAS cells than in wild-type cells (data not shown). Reporter gene activation was even suppressed in Hepa IPAS cells following transient overexpression of HIF-1 α , indicating that IPAS impairs productive interaction between HIF-1 α and the HRE.

HRE-specific DNA binding activity by the HIF-1 α /Arnt heterodimeric complex was lower in nuclear extracts from either normoxic or hypoxic IPAS-expressing cells than in corresponding nuclear extracts from wild-type cells (Fig. 3E). The dioxin (aryl hydrocarbon) receptor which mediates gene regulation in response to xenobiotic chemicals is also a member of the bHLH/PAS transcription factor family and shares the dimerisation partner factor Arnt with HIF-1 α ¹³. In contrast to hypoxia-inducible gene expression, dioxin-induced expression of dioxin receptor target gene cytochrome P-4501A1 was not perturbed in the Hepa IPAS cells (data not shown).

Six week-old C57Bl6/J healthy mice sacrificed by a lethal dose of CO₂ and their primary corneal epithelial cells were isolated. The eyes were enucleated and the corneal tissue was dissected in DME medium supplemented with 10% bovine calf serum under a stereomicroscope. Tissue masses were placed onto a gelatin-coated tissue culture plate and incubated in the same media supplemented with 3 ng/ml human recombinant FGF- β . After incubation for 8 days, corneal epithelial cells were trypsinised, and single cell suspensions were used for subsequent assays.

Example 3: IPAS Regulation of Transcriptional Responses to Hypoxia

Due to the structural similarity of IPAS to HIFs and the colocalization of IPAS and HIF-1 α in mouse cornea the putative role of IPAS in regulation of transcriptional responses to hypoxia was investigated. A transient transfection assay was performed in HeLa cells (ATCC) using a hypoxia-response element (HRE) driven luciferase reporter gene in the absence or presence of transiently expressed IPAS. In the absence of IPAS, incubation of the cells under hypoxic (1% O₂) conditions resulted in induction of reporter gene activity, reflecting the induced transactivation function of endogenous HIFs. Transient expression of IPAS reduced this hypoxia-inducible activation response (Figs. 3A, 3B).

Hypoxia-dependent activation of the reporter gene by coexpression of either HIF-1 α (Fig. 3A) or HLF/EPAS1 (Fig. 3A) was impaired in a dose-dependent manner by IPAS. IPAS acts as a dominant negative regulator of HIF-1 α and HLF/EPAS1-mediated gene expression. IPAS had no effect on hypoxia-induced protein stabilization of HIF-1 α and HLF⁷⁻¹⁰ (Fig. 3C) indicating that IPAS targeted regulatory steps located further down-stream than protein stabilization in HIF-mediated signal

transduction.

Example 4: RNA Blot and *in situ* Hybridization Analysis

Poly(A)⁺RNA samples (4.5 µg) were collected from a variety of C57B16 mouse tissues, Hepalcl7 and Hepa IPAS cells using the guanidiumthiocyanate method. The samples were purified using oligodT (Dyna). The purified samples were analyzed by northern blot analysis using ³²P-labeled cDNA probes of mIPAS (nt 623-897), mPGK1 (nt 426-771), mVEGF3 (nt 24-466), mCYP1A1 (nt 874-1199), and mβ-actin (nt 930-1075).

The results of the analysis of mouse tissue samples demonstrated that IPAS was expressed predominantly in the eye and at lower levels in the cerebellum and the cerebrum. No expression was detected in other tested mouse tissues, indicating a tissue-restricted expression pattern of IPAS mRNA (Fig. 1C).

In situ hybridization assays of tissue samples from 8 week-old C57B16 mice were performed to characterize the spatial expression pattern of IPAS in the eye and cerebellum. The in situ hybridizations were conducted using ³⁵S-labeled mIPAS or mHIF-1α antisense RNA probes according to known methods²³.

Intense IPAS expression was observed in the epithelial cell layer of the cornea (Figs. 2A, 2B) and with lower intensity in the layers of ganglion cells, inner nuclear cells, and rods and cones of the retina (Figs. 2E, 2F). Expression of HIF-1α mRNA was detected at low levels in the epithelium of the cornea (Figs. 2C, 2D), demonstrating dominant expression of IPAS over HIF-1α in these cells. HIF-1α was also expressed in the same layers of retina where IPAS expression was observed (Figs. 2G, 2H). In the cerebellum, expression of IPAS was limited to the Purkinje cell layer (Figs. 2I, 2J). HIF-1α did not show any similar spatially defined expression throughout the sections (Figs. 2K, 2L). Both IPAS and HIF-1α mRNAs were detected as weak diffuse signals over nonspecific background levels in certain areas of the cerebrum (data not shown).

Example 5: Electrophoretic Mobility Shift and *in vitro* Protein Interaction Assays

Nuclear extracts from either normoxic or hypoxic cells were prepared and analyzed by electrophoretic mobility shift assays as known in the art¹⁶. GST-IPAS or

GAL4 fusion proteins spanning various fragments of HIF-1 α were generated by translation either in the presence or absence of ^{35}S -labeled methionine in rabbit reticulocyte lysate (Promega). Equal concentrations of ^{35}S -labeled, *in vitro* translated Arnt, HIF-1 α or IPAS were incubated with GST-IPAS or GAL4-HIF-1 α fusion proteins for 1 hour at room temperature. Afterward, the proteins were incubated with anti-GST (Amersham-Pharmacia Biotech) or anti-GAL4 (Upstate Biotechnology) antibodies coupled to Protein A-Sepharose (Amersham-Pharmacia Biotech) for 1 hour at room temperature. After brief centrifugation, coimmunoprecipitated proteins were analyzed by SDS polyacrylamide gel electrophoresis and autoradiography.

Example 6: Assays for IPAS Interaction with HIF-1 α or Arnt

Coimmunoprecipitation assays were conducted to examine whether the inhibitory action of IPAS was mediated by direct interaction with HIF-1 α or Arnt. Glutathione S-transferase- (GST-) IPAS fusion protein was coprecipitated with HIF-1 α but not with Arnt, demonstrating specific physical interaction between IPAS and HIF-1 α (Fig. 3F). The assays further demonstrated that the N-terminal structures of HIF-1 α (amino acids 1-330, mainly composed of the bHLH/PAS motif) mediated association with IPAS (Fig. 3G).

Mammalian two-hybrid assays employing a GAL4 fusion protein spanning the bHLH/PAS domain of HIF-1 α as a bait demonstrated interaction with an IPAS-VP16 fusion protein. Consistent with the *in vitro* precipitation experiments, GAL-4-IPAS failed to show any interaction with Arnt-VP16 *in vivo* (data not shown). The IPAS/HIF-1 α complex was inert with regard to binding to an HRE probe. The HRE binding activity of HIF-1 α /Arnt heterodimer was impaired upon exposure to IPAS (Fig. 3H). Both of these results are consistent with the reduced levels of HIF-1 α -dependent DNA binding activity observed in nuclear extracts of cells stably expressing IPAS (Fig. 3E).

Example 7: Investigation of Role of IPAS in Corneal Epithelium

The cornea is an interesting study target because the transparency required for clear vision is present only with total avascularity. It is known that hypoxic conditions exist in the corneal environment following overnight closure of the eye during

sleep^{14,15}. The levels of hypoxia reached in the cornea would be sufficient to stimulate HIF-1 α -dependent gene expression in other cell types. However, such a gene activation response and neovascularization of the cornea do not normally occur, suggesting a mechanism of negative regulation.

Analysis of VEGF mRNA expression in primary cultures of mouse cornea epithelium cells was conducted to ascertain whether IPAS plays such a role in negative regulation of VEGF expression in the cornea. A low but detectable level of IPAS mRNA expression under normoxic conditions was detected. Exposure of the cells to hypoxia for 24 hours resulted in enhanced IPAS mRNA expression levels (Fig. 5A).

Non-corneal mice tissues were exposed to hypoxia (6% O₂) for 6 hours to assess hypoxia-induced IPAS expression regulation. Consistent with other experimental results (Fig. 1C), very low levels of IPAS expression were observed in most tissues of control mice kept at normoxia. In addition to corneal cells, IPAS mRNA expression was induced by hypoxia (2.2- to 3.9-fold induction) in the cerebrum, cerebellum, heart, and skeletal muscle (Fig. 5B), suggesting IPAS may modulate hypoxia-inducible gene regulatory responses in these tissues under conditions of hypoxia or ischemia.

In contrast to wild-type mouse Hepalclc7 cells¹⁶, primary cornea cells were transfected with an empty expression vector and demonstrated almost undetectable basal levels of VEGF mRNA expression. Hypoxia produced only a very modest induction response (Fig. 5C).

Primary cornea cells were transiently transfected with an antisense IPAS expression vector to down-regulate IPAS expression levels. The introduction of the antisense IPAS vector resulted in elevation of basal VEGF mRNA expression levels and significant hypoxia-inducible expression of the VEGF gene (Fig. 5C), showing IPAS has an important role in negative regulation of both basal level expression and hypoxia-dependent activation of the VEGF gene in cornea epithelium.

Example 8: Mouse Corneal Micropocket Assay and Hypoxic Exposure of Mice

The mouse corneal angiogenesis assay was performed as previously reported²⁴. Slow release micropellets of sucrose aluminium sulfate and hydron polymers

containing 2.5 µg of phosphorothioate-coupled IPAS antisense (5'-TCACGCGCTG CAGCCCCAAC GCCAT-3') (SEQ ID NO:3), sense (5'-ATGGCGTTGG GGCTGCAGCG CGTGA-3') (SEQ ID NO:4), or scrambled oligonucleotides were implanted into corneal pockets of 6 week-old male C57Bl6/J mice. The full length IPAS antisense sequence is provided in SEQ ID NO:5. Alternate sequences of these or any other disclosed or claimed sequence may be employed. Alternate sequences include functional fragments and analogs, or entities having acceptable levels of homology with the specific sequence described. Construction and use of alternate sequences is known in the art.

Six mice and 12 corneas were used in each group. The vascular area of the corneas of all the mice was measured by slit-lamp stereomicroscopy on day 5 after pellet implantation, and statistically evaluated by Student's two-tailed t-test. In hypoxia experiments eight week-old mice were either kept at normoxia or exposed to normobaric hypoxia (6% O₂) for 6 hours in an airtight chamber flushed with a mixture of nitrogen and room air.

Using similar methods, an analysis of the stimulation of blood vessel growth from the limbus region was recorded five normoxic days after implantation of micropellets. Significant ($p < 0.001$ compared to sense or scrambled oligonucleotide controls) induction of neovascularization in the corneas of mice treated with the IPAS antisense oligonucleotide (Figs. 5D, 5E) was observed. The physiological conditions of hypoxia which occur in the cornea, e.g., during sleep^{14,15}, can therefore induce vascular growth upon inhibition of IPAS expression.

Example 9: Tumor Studies in Mice

Approximately 1×10^6 wild-type Hepalclc7 or stably IPAS-expressing hepatoma cells were implanted subcutaneously in an 8-10 week old male immunoincompetent SCID mouse. Six mice were in the treated group and seven mice were in the control group. Primary tumors were measured using digital calipers on the days indicated. Tumor volumes were calculated according to the formula: width² x length x 0.52 as is common in the art²⁴. At day 18 after implantation, tumor tissues were resected and fixed with 4% formalin in phosphate-buffered saline for 24 hours. Tissues were imbedded in paraffin and stained with a rat anti-mouse antibody against

CD31 antigen²⁴. Positive signals were counted under a light microscope in at least 6 random fields at 40x magnification.

The role of IPAS in negative regulation of angiogenesis was evaluated by determining both the growth rates and vascular density of the tumors formed following implantation. As shown in Fig. 4A, IPAS-expressing hepatoma cells produced tumors which showed a markedly slower growth rate than that of the wild-type cells ($p < 0.001-0.01$ at all time points of observation). The tumors derived from the IPAS-expressing cells showed a significantly ($p < 0.001$) reduced vascular density in comparison to the wild-type tumors, as assessed by staining tumor samples with CD31 antibodies (Figs. 4B, 4C).

Example 10: IPAS as a Pro-Angiogenic Treatment

An organism would be treated with antisense IPAS by any means appropriate and, if necessary, including any pharmaceutically acceptable carrier or adjuvant. Treatment may include such methods as topical application, injection, infusion, gene therapy, use of liposomes or controlled release mechanisms, and a variety of any and all other methods determined appropriate by the practitioner. Antisense IPAS would effectively inactivate IPAS and prevent conditional negative regulatory strategy. This would occur because IPAS would be prevented from forming a nonfunctional complex with HIF-1 α . Such a mechanism is depicted in Fig. 5F. The mechanism relied upon for the present invention and depicted in Fig. 5F resembles the mechanism of repression of the function of MyoD and related bHLH differentiation factors by Id²⁰.

Treated regions of the organism may preferably include tissues exposed to hypoxia, such as the cornea or ischemic heart tissue. Following treatment, HIF-1 α activity would no longer be suppressed and angiogenesis would proceed. Conditions that may benefit from treatment with IPAS antagonists according to the present invention include, but are not limited to, coronary heart disease, wound healing, stroke, and diabetic ulceration.

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24. Cao, R. *et al.* Suppression of angiogenesis by a novel inhibitor, K1-5, generated by plasmin-mediated proteolysis. *Proc. Natl. Acad. Sci. USA* 96, 5728-5733 (1999).

WHAT IS CLAIMED IS:

1. A pharmaceutical composition, comprising the sequence of SEQ ID NO:3, or active fragments or analogs thereof.
2. A pharmaceutical composition, comprising the sequence of SEQ ID NO:4, or active fragments or analogs thereof.
3. A pharmaceutical composition, comprising the sequence of SEQ ID NO:5, or active fragments or analogs thereof.
4. A pharmaceutical composition, comprising a sequence complimentary to the sequence of SEQ ID NO:1, or active fragments or analogs thereof.
5. A pharmaceutical composition, comprising a sequence having 95% homology to the sequence of SEQ ID NO:5.
6. A pharmaceutical composition, comprising a sequence having 90% homology to the sequence of SEQ ID NO:5.
7. A pharmaceutical composition, comprising a sequence having 85% homology to the sequence of SEQ ID NO:5.
8. A pharmaceutical composition, comprising a sequence having 80% homology to the sequence of SEQ ID NO:5.
9. A pharmaceutical composition, comprising a sequence having 75% homology to the sequence of SEQ ID NO:5.
10. A pharmaceutical composition, comprising a sequence having 70% homology to the sequence of SEQ ID NO:5.

11. A pharmaceutical composition according to any one of Claims 1-10, further comprising at least one item selected from the group consisting of pharmaceutical carriers, adjuvants, and diluents.
12. A method of increasing angiogenesis in a cell, a group of cells, or an organism, comprising administering a pharmaceutically effective amount of a composition according to any one of Claims 1-10 to the cell, group of cells, or organism.
13. A method of increasing HIF-1 α function in a cell, a group of cells, or an organism, comprising administering a pharmaceutically effective amount of a composition according to any one of Claims 1-10 to the cell, group of cells, or organism.
14. A method according to any one of Claims 12 and 13, wherein the cell, group of cells, or organism are under hypoxic conditions.
15. A method of treating a hypoxia-related condition in a cell, a group of cells, or an organism, comprising administering a pharmaceutically effective amount of a composition according to any one of Claims 1-10 to the cell, group of cells, or organism.
16. The method of Claim 15, wherein the hypoxia-related condition is selected from the group consisting of ischemia, coronary heart disease, wound healing, stroke, and diabetic ulceration.
17. A method of maintaining normal cell or tissue function under hypoxic conditions, comprising administering a pharmaceutical composition according to any one of Claims 1-10 to the cell or tissue.
18. A plasmid comprising the sequence of SEQ ID NO:3.
19. A plasmid comprising the sequence of SEQ ID NO:4.

20. A plasmid comprising the sequence of SEQ ID NO:5.
21. A plasmid comprising a sequence complimentary to the sequence of SEQ ID NO:1.
22. A vector comprising a plasmid according to any one of Claims 18-21.
23. A host cell transfected by a vector according to Claim 22.
24. A host cell expressing the protein encoded by any one of SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5.

Fig. 1

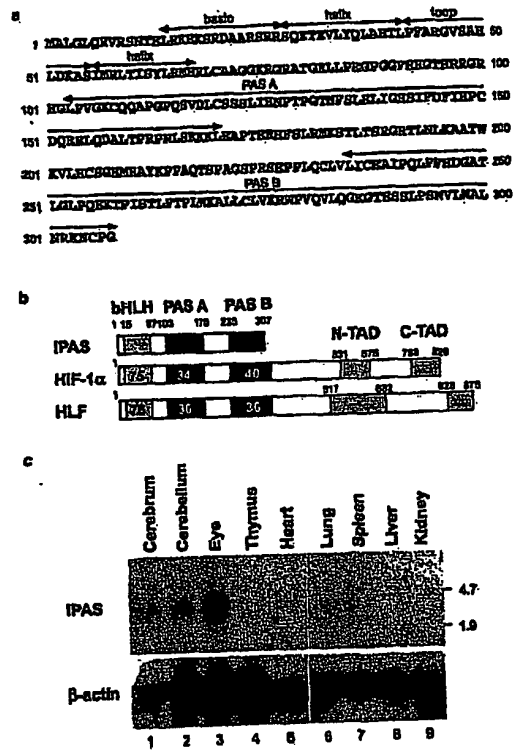


Fig. 2

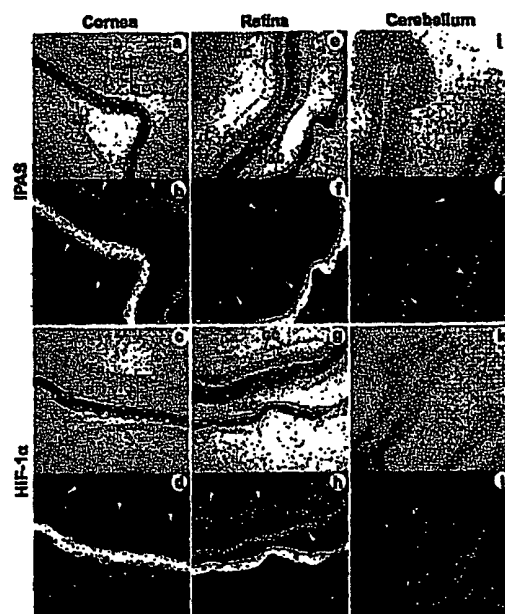


Fig. 3

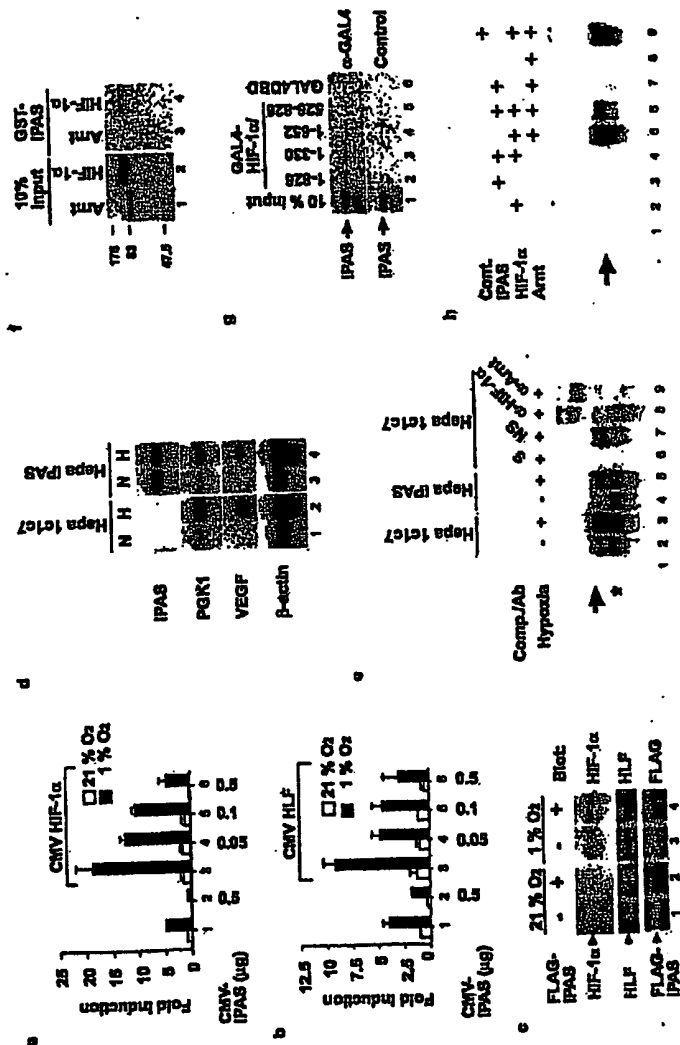


Fig. 4

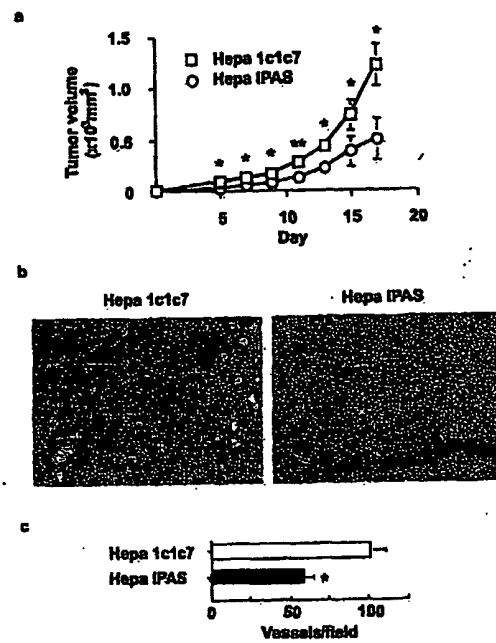


Fig. 5

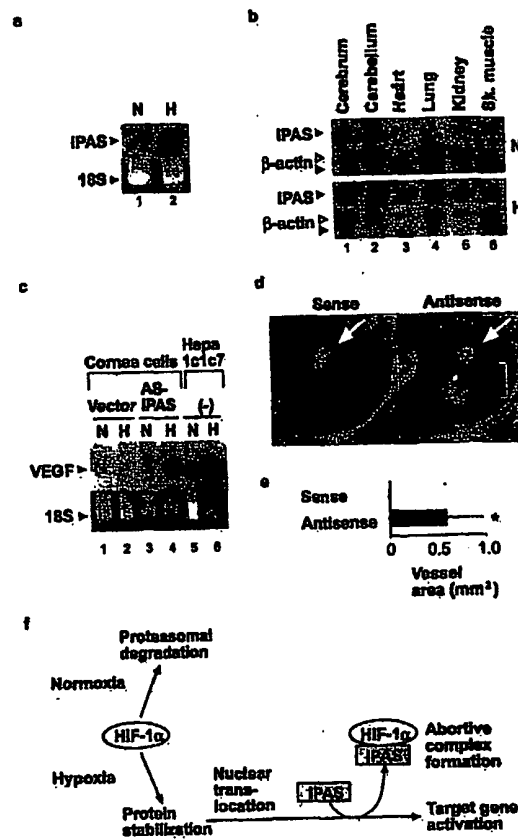


Fig. 6

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151 GTCAGCGCGCACCTGGACAAGGCCTCCATCATGCGCCTCACAATCAGCTA
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601 GCGGCCACCTGGAAGGTGCTGCACTGCTCAGGACATATGAGGGCCTACAA
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SEQUENCE LISTING

<110> MAKINO, Yuichi
CAO, Yihai
POELLINGER, Lorenz

<120> REGULATION OF HYPOXIA-INDUCIBLE GENE EXPRESSION WITH ANTISENSE
INHIBITORY PAS DOMAIN PROTEIN

<130> 056187.50678WO

<140> to be assigned

<141> 2002-11-28

<150> US 60/333,513

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Arg	Met	His	Arg	Leu	Cys	Ala	Ala	Gly	Gly	Lys	Arg	Gly	Arg	Ala	Thr	65	70	75	80
Gly	Arg	Leu	Leu	Pro	Glu	Gly	Pro	Gly	Gly	Phe	Arg	His	Gly	Thr	His	85	90	95	
Arg	Arg	Gly	Arg	His	Gly	Leu	Pro	Val	Gly	Lys	Cys	Gln	Gln	Ala	Pro	100	105	110	
Gly	Pro	Gln	Ser	Val	Asp	Leu	Cys	Ser	Ser	Ser	Leu	Ile	His	Asn	Pro	115	120	125	
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2/4

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Arg Ser Glu Pro Pro Leu Gln Cys Leu Val Leu Ile Cys Glu Ala Ile
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Lys Thr Pro Ile Ser Thr Leu Phe Thr Pro Leu Trp Lys Ala Leu Leu
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Cys Leu Val Lys Arg Trp Pro Val Gln Val Leu Gln Gly Lys Gly Thr
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 02/02198

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: A61K 48/00, C12N 15/11, C07K 14/47, A61K 38/16
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: A61K, C12N, C07K, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	US 20020165140 A (ANDERS BERKENSTAM ET AL), 7 November 2002 (07.11.02), page 2, column 2, line 5 - line 18, claims 8,9 --	1-24
A	WO 0009657 A2 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE), 24 February 2000 (24.02.00), page 1, line 34 - page 3, line 19; page 5, line 25 - page 6, line 23; page 7, line 7 - line 15, page 15, line 19 - line 23; abstract --	1-24

☒ Further documents are listed in the continuation of Box C.
 ☒ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search	Date of mailing of the international search report
4 March 2003	02 -04- 2003
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Malin Söderman/Els Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 02/02198

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 0074725 A1 (DANA-FARBER CANCER INSTITUTE, INC.), 14 December 2000 (14.12.00), page 4, line 22 - page 5, line 7; page 9, line 3 - page 12, line 11; page 15, line 10 - line 13, abstract --	1-24
A	WO 9856936 A1 (MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V.), 17 December 1998 (17.12.98), page 11, line 17 - line 20; page 24, line 10 - line 22, abstract --	1-24
A	WO 9928469 A1 (GENZYME CORPORATION), 10 June 1999 (10.06.99), page 3, line 3 - line 24; page 29, line 11 - line 20 --	1-24
X	WO 0181581 A2 (CORIXA CORPORATION), 1 November 2001 (01.11.01), WO 0181582 & Database EMBL (on line Accession no. AAU45894 27 Feb 2002, retrieved from EBI, 100% identity in 5aa overlap with SEK NO 3	1
X	27 Feb 2002, 100% identity in 5aa overlap with SEK ID ID NO 4 --	2
P,X	WO 0202609 A1 (BIOVITRUM AB), 10 January 2002 (10.01.02), WO 200202609 A & Accession no ABK 14502 08 May 2002, retrieved from EBI 100% identity in 25 nt overlap with SEQ ID NO 3 --	1-24
P,X	J. Biol. Chem., Vol. 277, No 36, September 2002, Yuichi Makino et al: "Inhibitory PAS Domain Protein (IPAS) Is a Hypoxia-inducible Splicing Variant of the Hypoxia-inducible Factor-3 alpha Locus", pages 32405 - 32408, page 2, line 18-28, AF 481147, 14 Mar 2002, 96,296 % Identity in 54 nt overlap with SEQ ID NO 5 --	1-24

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 02/02198

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9928464 A2 (WISCONSIN ALUMNI RESEARCH FOUNDATION), 10 June 1999 (10.06.99), WO 9928464, page 29, line 13-19, AAX 58986, 23 Aug 1999, 79, 7751 identity in 89 nt overlap with SEQ ID NO 5, 76, 316% identity in 304aa overlap with SEQ ID NO 1	9,10
A	Acc no AAX50986 --	1-8,11-24
A	WO 9928464 A2 (WISCONSIN ALUMNI RESEARCH FOUNDATION), 10 June 1999 (10.06.99), AAY06295, 23 Aug 1999	1-8,11-24
X	76.596% identity in 235 aa overlap with SEQ ID NO 1 --	9-10
P,X	Nature, volume 414 (6863) 2001, Makino Y. et al: "Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression", pages 550-554, AF 416641, 11 Dec 2001, 79,775 % identity in 89 nt overlap with SEQ ID NO 5, Acnr AF416641, 11 Dec 2001, 100% ID in 307aa overlap with SEQ ID NO 1, Accnr Q8VHRI, 1 Mar 2002, 100% identity in 307 aa overlap SEQ 10 No 1 --	1-24
P,X	Artikel ref, Accession nr AF416641, 11 Dec 2001, 100 % identity in 25 nt overlap with SEQ ID NO 3. --	1-24
A	Database Acc No AA028416, 100 % ID in 51 nt overlap with SEQ No 5, 17 Aug 1996, - Database Acc No A1322407, 29 Dec 1998, 100 % ID In 25 nt overlap with SEQ ID No 3 - Acc No AI 322401, 29 Dec 1998, 100 % identity in 25 nt overlap with SEQ ID NO 4 AI 322407, 29 Dec 1998, 100 % identity in 152 aa overlap with SEQ ID No 1 --	1-24

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 02/02198

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Q 9RQ54, May 2000, 76.596 % identity in 235 an overlap with SEQ ID NO 1 AF 079143, 02 Jan 2000, 100% identity in 50 nt overlap with SEQ ID NO 5 --	1-24
X	WO 0159063 A2 (HUMAN GENOME SCIENCES, INC.), 16 August 2001 (16.08.01), ABA-14358, 23 Jan 2002, 92,727 % identity in 55 nt overlap with SEQ ID NO 5 --	6-10
P,X	WO 0202609 A1 (BIOVITRUM AB), 10 January 2002 (10.01.02) --	1-24
P,X	WO 200202609 A & Accession no ABK 14502 08 May 2002, retrieved from EBI, 100% identity in 25 nt overlap with SEQ ID NO 4 Acc no: AAU 75902, 8 May 2002 100% identity in 307 aa overlap with SEQ ID NO 1 Acc no: ABK 14502, 8 May 2002 100% identity in 307 aa overlap with SEQ ID NO 1 Acc no: ABK 14501, 8 May 2002 100% identity with SEQ ID NO 5 -- -----	1-24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE02/02198**Box I** Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: **12-15, 17**
because they relate to subject matter not required to be searched by this Authority, namely:
see next sheet
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

Claims 12-15,17 relate to methods of treatment of the human or animal body by surgery or by therapy/diagnostic methods practised on the human or animal body/Rule 39.1.(iv). Nevertheless, a search has been executed for these claims. The search has been based on the alleged effects of the compounds/compositions.

INTERNATIONAL SEARCH REPORT

Information on patent family members

30/12/02

International application No.

PCT/SE 02/02198

Patent document cited in search report			Publication date	Patent family member(s)		Publication date
US	20020165140	A	07/11/02	NONE		
WO	0009657	A2	24/02/00	AU	5562999 A	06/03/00
				US	6395548 B	28/05/02
				WO	0010096 A	24/02/00
WO	0074725	A1	14/12/00	EP	1198255 A	24/04/02
WO	9856936	A1	17/12/98	AU	8536998 A	30/12/98
				EP	0990042 A	05/04/00
WO	9928469	A1	10/06/99	AU	749467 B	27/06/02
				AU	1626899 A	16/06/99
				CA	2311643 A	10/06/99
				EP	1034267 A	13/09/00
				IL	136454 D	00/00/00
				JP	2001525173 T	11/12/01
				NO	20002849 A	02/08/00
				US	6432927 B	13/08/02
				US	6274585 B	14/08/01
WO	0181581	A2	01/11/01	AU	5552401 A	07/11/01
				EP	1278854 A	29/01/03
WO	0202609	A1	10/01/02	AU	2418001 A	25/06/01
				AU	7477201 A	14/01/02
				SE	0002551 D	00/00/00
				US	2002165140 A	07/11/02
WO	9928464	A2	10/06/99	AU	1610499 A	16/06/99
WO	9928464	A2	10/06/99	AU	1610499 A	16/06/99

INTERNATIONAL SEARCH REPORT

Information on patent family members

30/12/02

International application No.

PCT/SE 02/02198

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO	0159063	A2	16/08/01	AU 2950801 A 07/08/01
				AU 3095801 A 07/08/01
				AU 3645901 A 07/08/01
				AU 3646001 A 07/08/01
				AU 3646101 A 07/08/01
				AU 3646201 A 07/08/01
				AU 3646301 A 07/08/01
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